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<b>(54) Title:</b> CARBONIC ANHYDRASE ISOENZYME  <b>(57) Abstract</b>  Essentially pure carbonic anhydrase VI, a protein isolated from saliva and the parotid gland. Sub-units or fragments of carbonic anhydrase VI. A process for the isolation of carbonic anhydrase VI.		

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#### CARBONIC ANHYDRASE ISOENZYME

The present invention relates to a carbonic anhydrase isoenzyme and to processes for its purification to homogeneity.

[Note: Literature references cited herein are given in

5 full at the end of the specification]

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) which catalyses the reversible hydration of carbon dioxide (1) has been isolated from a wide range of species and tissues (2, 3, 4). From mammalian  
10 tissues, three isoenzymes of carbonic anhydrase have been well characterized: carbonic anhydrase I, a low activity isoenzyme which occurs primarily in erythrocytes; carbonic anhydrase II, the high activity isomer which has a wide tissue distribution and  
15 carbonic anhydrase III which is present mainly in red skeletal muscle (5). The three isoenzymes are the products of three different genes (6). All are monomeric proteins with molecular weights of about 30,000 containing one zinc atom per molecule. Another

group of carbonic anhydrases which are not as well characterized are the membrane-bound enzymes. They appear to differ from the other isoenzymes in a number of properties. These membrane bound enzymes have been  
5 classified carbonic anhydrase IV. These detergent-solubilized proteins have been difficult to purify which explains the paucity of data relating to them (7). However it is known that they have higher molecular weights than the other isoenzymes. For  
10 example, human renal carbonic anhydrase IV has a molecular weight of 68,000 (7) and bovine lung carbonic anhydrase IV 52,000 (8). At least part of this appears to be due to the presence of carbohydrate. These isoenzymes also have amino acid  
15 compositions different from those of the other isoenzymes. More recently (9) another type of carbonic anhydrase has been purified from the hepatocytes of guinea-pig mitochondria and this has been designated carbonic anhydrase V.

20 In 1979, we reported the presence of a high molecular weight fraction from sheep parotid gland and saliva which possessed carbonic anhydrase activity (10). The high molecular weight fraction accounted for about 40% of the total carbonic anhydrase activity  
25 of the parotid gland, the rest being due to the carbonic anhydrase II isoenzyme (10). The high molecular weight fraction was not purified to homogeneity or characterized in detail.

A high molecular weight fraction of rat saliva  
30 possessing carbonic anhydrase activity has been reported (11). The carbonic anhydrase component of this fraction was not well characterized.

We have now found and purified to homogeneity a carbonic anhydrase isoenzyme isolated from sheep

saliva and salivary glands. This enzyme appears to be unique to the salivary glands and because it is secreted into the saliva, it forms a new class of carbonic anhydrase isoenzymes, hereinafter referred to as "carbonic anhydrase VI". The purification to homogeneity of carbonic anhydrase VI has allowed characterisation of this protein, including determination of part of the N-terminal protein sequence.

10 According to the present invention, there is provided substantially pure carbonic anhydrase VI.

According to a further aspect of the present invention, there is provided fragments or sub-units of carbonic anhydrase VI, having carbonic anhydrase VI activity. Carbonic anhydrase activity is defined as the reversible hydration of carbon dioxide. Assays for carbonic anhydrase activity are well known, and are described, for example, by Fernley et al. (10).

The isolation and purification of carbonic anhydrase VI will enable investigations to be carried out to characterise its role in the maintenance of saliva pH. Antibodies raised against the sheep enzyme cross-react with the human enzyme so its levels in human saliva can be measured readily. This will be important to clinical cases such as hypogeusia (decreased taste acuity) to determine if lack of this protein is the cause of this disease. If as expected, this protein plays a role in the mechanism of taste it will be possible, knowing the structure, to modify the protein or synthesize parts of it which may enhance taste perception. Thus it is possible that products derived from it could be used as flavour enhancers.

The purification of carbonic anhydrase VI and determination of its amino acid sequence thereof will

enable the production of synthetic oligonucleotides which corresponding to the amino acid sequence of carbonic anhydrase VI. Such oligonucleotides may be used as hybridization probes, for the isolation of the  
5 gene(s) encoding carbonic anhydrase VI.

According to a still further aspect of the present invention, there is provided allelic variants of carbonic anhydrase VI. These naturally occurring allelic variants may include amino acid differences in  
10 the overall sequence, or deletions, substitutions, insertions, or additions of an amino acid(s) of the sequence of carbonic anhydrase. Allelic variants may be produced according to the processes of the present invention.

15 According to another aspect of the present invention there is provided a process for the isolation of carbonic anhydrase VI which comprises the steps of:

- 20 (i) applying saliva or an extract from salivary glands to a first affinity resin capable of binding carbonic anhydrase VI, and eluting the bound material from the resin;
- (ii) applying the material eluted from the first affinity resin to a second affinity resin which  
25 binds carbonic anhydrase VI in a different manner to said first affinity resin, and eluting and recovering carbonic anhydrase VI from said second resin.

Affinity resins generally comprise a support  
30 matrix, such as sepharose 4B (trade mark, Pharmacia, Uppsala, Sweden), with protein binding groups attached to the matrix. The protein binding groups are selected so as to be capable of binding carbonic anhydrase VI, and preferably are selected from lectins

such as wheat germ lectin, antibodies directed against carbonic anhydrase VI or agents capable of binding to the active site of carbonic anhydrase VI, such as p-aminomethylbenzene sulfonamide.

- 5        If desirable, a size fractionation step may be performed on the carbonic anhydrase VI material recovered from the second affinity resin.

Extracts from salivary glands may be prepared by homogenising the glands in an appropriate buffer, such  
10 as phosphate buffer pH 7.4. Protein extracted during the homogenising step may be concentrated by precipitation, prior to loading onto an affinity resin.

Various aspects of the present invention will now be described by way of example only with reference  
15 to the accompanying drawings in which:

FIGURE 1 shows a plot of electrophoretic mobility of a number of well characterised protein against the log of their molecular weights. Phosphorylase a (M.W 94,000), bovine serum albumin  
20 (M.W 67,000), soyabean trypsin inhibitor (M.W 21,100) and  $\alpha$ -lactalbumin (M.W 14,400) were electrophoresed on 7.5% to 15% polyacrylamide gradient gels in 0.1% SDS. The gels were stained with Coomassie blue and the electrophoretic mobility determined. The  
25 electrophoretic mobility of carbonic anhydrase VI is represented by an open circle.

Inset: SDS polyacrylamide gel (7.5 - 15%) of purified carbonic anhydrase VI stained with Coomassie blue.

30        FIGURE 2 shows an HPLC analysis of cyanogen bromide digests of reduced and carboxymethylated carbonic anhydrase VI (upper panel) and carbonic anhydrase II (lower panel). Absorbtion as 214 nm is plotted against elution time.

FIGURE 3 shows the amino acid sequence of carbonic anhydrase VI. The amino acids are represented by the one-letter code as defined by the IUPAC-IUB Joint Commission on Biochemical Nomenclature [Biochem. J. (1982) 219, 345-373]. The carbohydrate attachment sites are designated by the asterisks;

FIGURE 4 shows a western analysis of various tissues of the sheep using anti-ovine carbonic anhydrase VI antibody. Sixteen different tissues from sheep were homogenized and ultracentrifuged [100,000 g, 60 min]. 3 µg of protein from each tissue (except 0.6 µg of parotid protein) was denatured in SDS and run on a 10.5% acrylamide -0.1% SDS gel. The proteins were electroblotted from the gel to nitrocellulose and this was probed with affinity-purified anti-ovine carbonic anhydrase VI antibody. The bands were visualized with a second antibody coupled to horse radish peroxidase (Biorad). Molecular mass markers were run on each gel and these are indicated on the side of each nitrocellulose sheet with the mass in kilo daltons (130, 75, 50, 39, 27 and 17).

Lane 1, 0.5 µg of CA II standard; Lane 2, kidney medulla; Lane 3, kidney cortex; Lane 4, sub-lingual; Lane 5, sub-mandibular; Lane 6, Molecular mass (Mr) markers; Lane 7, parotid; Lane 8, 0.5 µg CA VI standard; Lane 9, adrenal; Lane 10, abomasum; Lane 11, liver; Lane 12, lung; Lane 13, Mr Markers; Lane 14, pancreas; Lane 15, skeletal muscle; Lane 16, CA VI standard; Lane 17, CA II standard; Lane 18, small intestine; Lane 19, cortex of the brain; Lane 20, ovary; Lane 21, Mr markers; Lane 22, ventricle; Lane 23, atria; Lane 24, CA VI standard.

FIGURE 5 is a hybridization histochemical



analysis of sheep sub-mandibular cells, parotid gland and liver using an oligonucleotide based on the 19 amino-terminal amino acid residues of sheep carbonic anhydrase VI. An X-ray film autoradiograph is shown.

5       FIGURE 6 shows a western analysis of carbonic anhydrase (VI) from different species. CA VI was isolated from the saliva or parotid glands of several different species and 0.5 µg of each of the purified proteins was run on a 10.5% acrylamide - 0.1% SDS gel  
10 and electroblotted on to nitrocellulose. The nitrocellulose was reacted with affinity purified anti-ovine CA VI antibody and the antigen-antibody complexes were visualized by the horse radish peroxidase method (Biorad). Lane 1, ovine CA VI; Lane  
15 2, Molecular mass markers; Lane 3, bovine CA VI; Lane 4, human CA VI; Lane 5, rat CA VI; Lane 6, dog CA VI.

Abbreviations:

SDS-PAGE - Sodium dodecylsulphate polyacrylamide gel electrophoresis;

20 TRIS       - Tris(hydroxymethyl amino methane;)  
HPLC       - High performance liquid chromatography;  
SDS        - Sodium dodecyl sulphate.  
CA         - Carbonic anhydrase

Definitions:

25       The term "carbonic anhydrase VI" refers to an enzyme having carbonic anhydrase activity found in saliva, and having a sub-unit molecular weight of approximately 45,000 daltons as determined by SDS-PAGE and an amino terminal peptide sequence having the  
30 following sequence:

H-Gly-His-Gly-Val-Glu-Trp-Thr-Tyr-Ser-Glu-Gly-Met-  
Leu-Asp-Glu-Ala-His-Trp-Pro-Leu-Glu-Tyr-Pro-Lys-  
Cys-Gly---

Particularly, carbonic anhydrase VI refers to a protein having a protein sequence the same as or substantially corresponding to amino acids 1 to 307 of Figure 3.

- 5       The amino acid sequence of carbonic anhydrase VI may be varied from that depicted in Figure 3 by the substitution, addition, or deletion of one or more amino acids. Variants which catalyse the reversible hydration of carbon dioxide, characteristic of  
10 carbonic anhydrase VI, are included within the definition of carbonic anhydrase VI. Such variants or fragments thereof may be produced, for example, by solid phase peptide synthesis techniques (14).

- We have isolated carbonic anhydrase VI from the  
15 sheep. It is to be understood that carbonic anhydrase VI may be readily isolated from other species following the teaching of the present invention. Any such protein having a sub-unit molecular weight of approximately 45,000 daltons and an amino acid  
20 sequence exhibiting substantial homology with the sequence set out in Figure 3, may be included within the definition of carbonic anydrase VI.

- "Substantial homology" when used in the above context refers to at least 60% homology with amino  
25 acids 1 to 307 of Figure 3.

- The term "sub-unit or fragment" refers to a peptide having an amino acid sequence which is included within the amino acid sequence of Figure 3 and is unique to that sequence. Peptides having more  
30 than six amino acids are likely to be unique to carbonic anhydrase VI. In order to test whether a peptide is unique to its amino acid sequence may be compared with amino acid sequences on record in amino

acid sequence data banks such as the EMBL Data Base (compiled by the European Molecular Biology Laboratory), the Dayhoff Data base or the Gene Bank Data Base (compiled by the National Institutes of Health, U.S.A.). Sub-units or fragments of carbonic anhydrase VI may or may not possess biological activity.

"Essentially pure" refers to carbonic anhydrase VI substantially free of protein or other contaminant material; generally wherein 95 percent of the total protein is carbonic anhydrase VI.

#### Methods:

##### Western Gel Analysis:

Protein from tissue samples was denatured in SDS and run on a 10.5% acrylamide - 0.1% SDS gel. The proteins were electroblotted from the gel to nitrocellulose, which was probed with affinity purified anti-ovine carbonic anhydrase VI antibody. The bands were visualised with a second antibody coupled to horse radish peroxidase (Biorad.).

##### Antisera Production:

Antibodies were raised by injecting rabbits intra-muscularly with the purified antigen mixed with Freund's adjuvant. The rabbits were injected every four weeks and bled from an ear vein six weeks after the first injection and subsequently every four weeks. The presence of CA antibody was tested by immuno-diffusion against pure CA VI.

##### Atomic Absorption Spectroscopy:

Zinc content of the ovine CA VI was measured on a Perkin-Elmer 272 atomic absorption spectrometer.

##### Amino Acid Sequence Determination:

The CA VI protein and peptides derived from CA VI were sequenced on an Applied Biosystems Inc. 470A protein sequencer with an on-line 120A

phenylthiohydration analyser.

Hybridization Histochemistry:

Six micron sections of tissues were made, and were hybridized with a  $^{32}\text{P}$ -labelled

5 oligodeoxyribonucleotide probe, according to the method of Coghlan et al. (12).

Oligonucleotide Production:

Oligodeoxyribonucleotide probes were synthesized by the solid phase phosphoramidite procedure (13) in an Applied Biosystems Inc. Model 380A DNA Synthesizer. The probes were purified by polyacrylamide gel electrophoresis. The sequence of the 56 mer probe used for hybridization histochemistry was as follows:

15 5'-GGCCAGTGGGCCTCGTCCAG  
CATGCCCTCGGAGTAGGT  
CCACTCCACGCCGTGGCC-3'

EXAMPLE 1

Purification of Carbonic Anhydrase VI

20 Carbonic anhydrase affinity resin was made by coupling 1 g (4.5 mmol) of p-aminomethylbenzene sulfonamide.HCl to 15 g of activated CH-Sepharose 4B (Pharmacia) using the procedure recommended by the manufacturer.

25 Parotid salivary glands were excised from merino and merino cross-bred sheep immediately following death. The glands were trimmed of excess fat and either frozen and stored at  $-20^{\circ}$  or homogenized in 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and  
30 0.5 mM phenylmethylsulphonyl fluoride. All purification steps were carried out at  $4^{\circ}\text{C}$ . The homogenate was centrifuged (20,000 g for 20 min) and the supernatant filtered through glass wool to remove any solid fat. An equal volume of a saturated ammonium sulphate solution was added to the

supernatant and was stirred for 1 h. The suspension was centrifuged (10,000 g for 15 min) and the precipitate taken up in a minimal amount of 0.1 M ammonium bicarbonate and dialysed against this buffer  
5 overnight. Particulate matter was removed by centrifugation. Alternatively, parotid saliva was collected from merino and merino cross-bred sheep by cannulation of the parotid duct. The saliva was collected on ice with enzyme inhibitors (aprotinin 1  
10  $\mu\text{g}.\text{ml}^{-1}$  and benzamidine, 1  $\text{mg}.\text{ml}^{-1}$ ) present. The saliva was filtered and concentrated about 10-fold by ultrafiltration.

The saliva or salivary gland extracts were passed through a column of the sulphonamide affinity  
15 resin. Carbonic anhydrase VI binds to the resin, and unbound protein was washed off with 50 mM TRIS (pH 8.0) buffer. Non-specifically bound protein was removed by washing the column with 0.2 M sodium iodide in 0.1 M ammonium bicarbonate. Carbonic anhydrase VI  
20 was eluted from the column with 0.1 M potassium cyanide in 0.1 M ammonium bicarbonate. These fractions were concentrated by ultrafiltration and loaded on to a column of wheat germ lectin-Sepharose (Pharmacia) equilibrated in 50 mM phosphate buffer (pH  
25 7.4), 0.2 M NaCl. After unbound protein was washed from the resin, the carbonic anhydrase VI was eluted by a 100  $\text{mg ml}^{-1}$  solution of N-acetyl-D-glucosamine in 50 mM phosphate buffer (pH 7.4), 0.2 M NaCl. The eluted peak was chromatographed on a column of  
30 Sepharose 6B in 0.1 M ammonium bicarbonate.

#### EXAMPLE 2

##### Properties of Carbonic Anhydrase VI

Carbonic anhydrase VI eluted from the Sepharose 6B column is homogeneous by SDS acrylamide gel

electrophoresis. Under these conditions it has a sub-unit molecular weight of 45,000 (Fig. 1). By gel filtration on Sepharose 6B (Pharmacia) it has an apparent native molecular weight of 540,000. It contains N-linked carbohydrate and when this is removed by the enzyme N-glycanase, it has a sub-unit molecular weight of 36,000. By atomic absorption spectroscopy, the enzyme was found to contain one zinc atom per sub-unit as for other carbonic anhydrases.

Antibodies raised against carbonic anhydrase VI do not cross-react with the carbonic anhydrase II isoenzyme. Also the cyanogen bromide derived peptide maps (by HPLC) of these two isoenzymes are clearly different (Fig. 2). These parameters show that carbonic anhydrase VI is not closely related to the carbonic anhydrase II isoenzyme.

The complete amino acid sequence of sheep carbonic anhydrase VI has been determined by sequence analysis of the intact protein and of the cyanogen bromide and tryptic peptides derived from the intact protein and is shown in Fig. 3. It shows a 33% sequence identity with the sheep CA II isoenzyme. It has retained all the amino acid residues involved in the active site of carbonic anhydrases but differs at other positions which are conserved in all the known cytoplasmic carbonic anhydrases.

Carbonic anhydrase VI appears to be unique amongst carbonic anhydrases in having a disulphide bond (Cys 25 - Cys 207). It also has two carbohydrate groups N-linked at Asn-50 and Asn-239. The protein isolated (from 1 sheep) for sequence analysis showed some sequence variability with both methionine and valine occurring at position 63 and methionine and isoleucine at positions 297. From this information it

would appear that there are at least four alleles of carbonic anhydrase VI.

Analysis of sixteen different tissues of the sheep by the technique of western analysis using  
5 antibodies raised against carbonic anhydrase VI showed that carbonic anhydrase VI was found only in the salivary glands and nearly all of that in the parotid gland (Fig. 4). This was confirmed by hybridization histochemistry which showed that mRNA for carbonic  
10 anhydrase VI was found in the acinar cells of the parotid gland (Fig. 5). The oligonucleotide probe used corresponds to the 19 N-terminal amino acids of carbonic anhydrase VI, and was 56 nucleotides long.

A number of species have been examined for the  
15 present of carbonic anhydrase VI in saliva or parotid glands and, it has been found in all examined (sheep, rat, cow, dog, kangaroo and human). The anti-sheep carbonic anhydrase VI antibody cross-reacted with all of these enzymes except those of the rat (Fig. 6).

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- 45



## CLAIMS:

1. Essentially pure carbonic anhydrase VI.
2. Essentially pure carbonic anhydrase VI as claimed in claim 1, having the sequence  
H-Gly-His-Gly-Val-Glu-Trp-Thr-Tyr-Ser-Glu-Gly-Met-  
Leu-Asp-Glu-Ala-His-Trp-Pro-Leu-Glu-Tyr-Pro-Lys-  
Cys-Gly---.
3. Essentially pure carbonic anhydrase VI as claimed in claim 1 having the amino acid sequence set out in Figure 3
4. A peptide fragment or allelic variant of carbonic anhydrase VI as claimed in claim 3.
5. A process for the isolation of carbonic anhydrase VI which comprises the steps of:
  - (i) applying saliva, or an extract from salivary glands to a first affinity resin capable of binding carbonic anhydrase VI, and eluting the bound material from the column;
  - (ii) applying the eluted material from said first resin to a second affinity resin, which binds carbonic anhydrase VI in a different manner to said first affinity resin; and eluting and recovering carbonic anhydrase VI from said second column.
6. A method as claimed in claim 5 wherein said affinity resins comprise a support matrix having groups capable of binding to carbonic anhydrase VI attached thereto.

7. A process as claimed in claim 6 wherein the groups capable of binding to carbonic anhydrase VI are selected from wheat germ lectin and p-aminomethylbenzene sulfonamide.

8. A process as claimed in any one of claims 5 to 7 wherein the material carbonic anhydrase VI eluted from the second affinity resin is subjected to a size fractionation step on a suitable chromatographic matrix .

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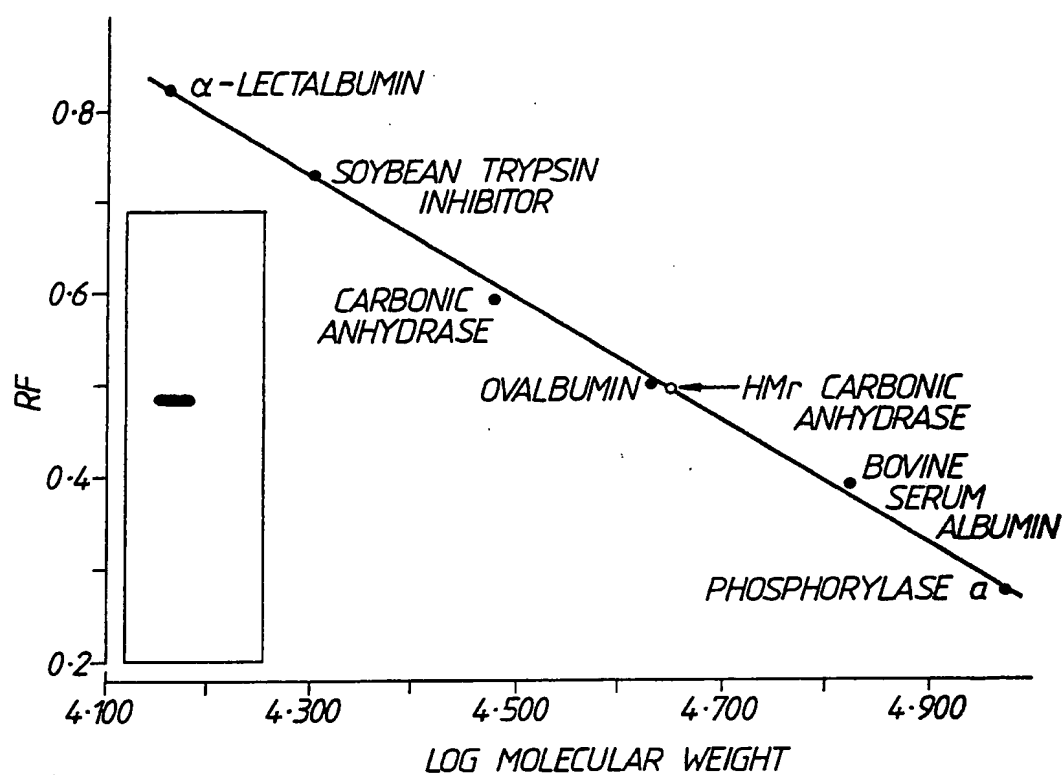


Fig.1.

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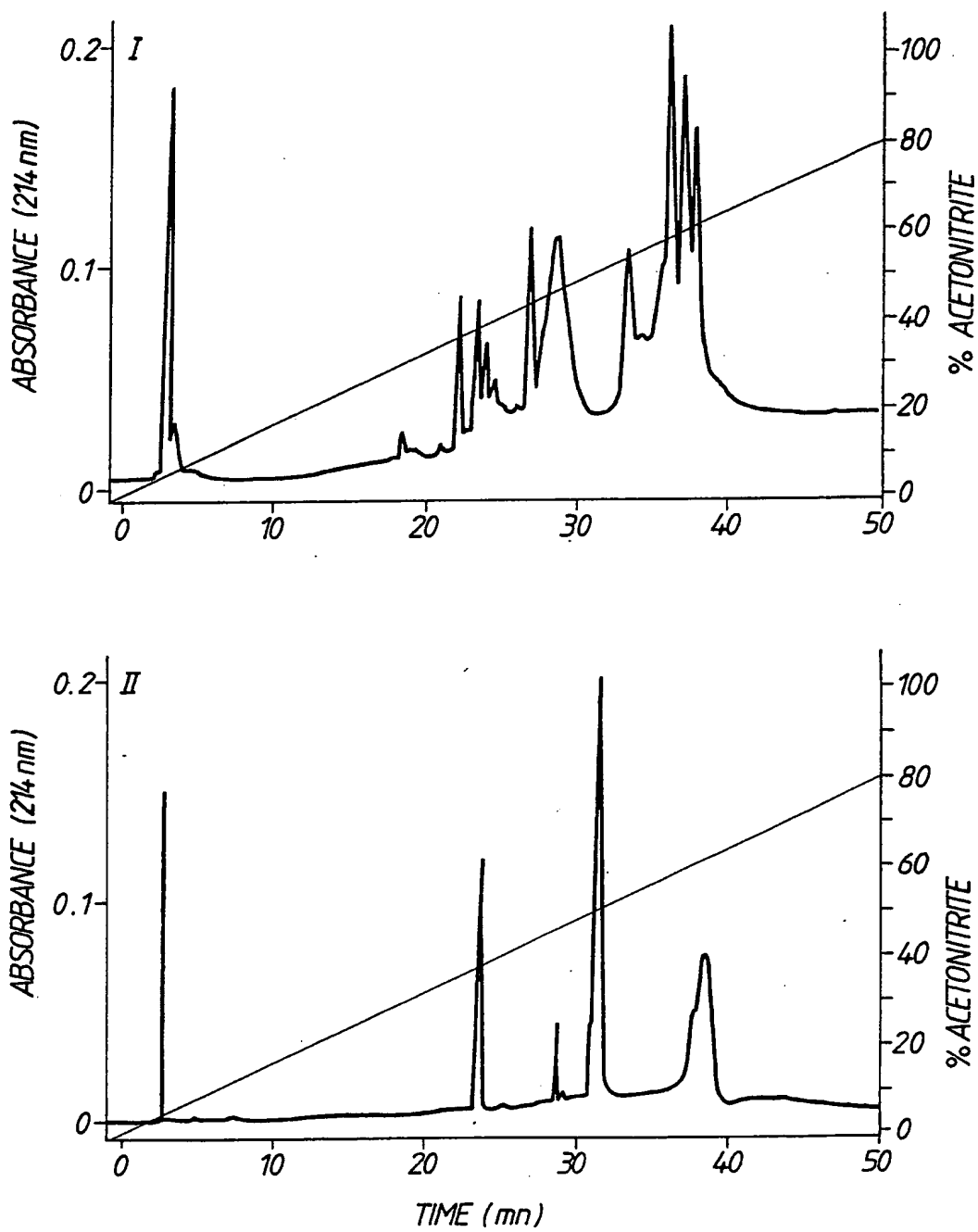


FIG.2.

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GHGVEW<sup>25</sup>TYSEGMLDEAHWPLEY<sup>50</sup>PKCGGRRQSPIDLQMKKVQYNPSLRALN<sup>\*</sup>

LTGYGLWHGEFP<sup>75</sup>—<sup>M</sup>TNNGHTVQISLPSTMSMTTSDGTQYLAKQMHHFWGGA<sup>100</sup>

SSEISGSEHTVDGMRYV<sup>125</sup>IEIHVVHYNSKYNSYEEAQKEPDGLAVLAALVE<sup>150</sup>

VKDYTENAYYSKFISHLEDIRYAGQSTVLRGLDIEDMLPGDLRYYSYLG<sup>200</sup>

SLTTPPCTENVHWFV<sup>225</sup>ADTVKLSKTQVEKLENSLLNHQNK<sup>\*</sup>TIQNDYRR<sup>250</sup>TQ

PLNHRVVEANFMSRPHQEY<sup>275</sup>TLASKLHFYLN<sup>I</sup>NIDQ<sup>300</sup>TLEYLRRFIEQK<sup>M</sup>—PKR

KKQENWP

Fig.3.

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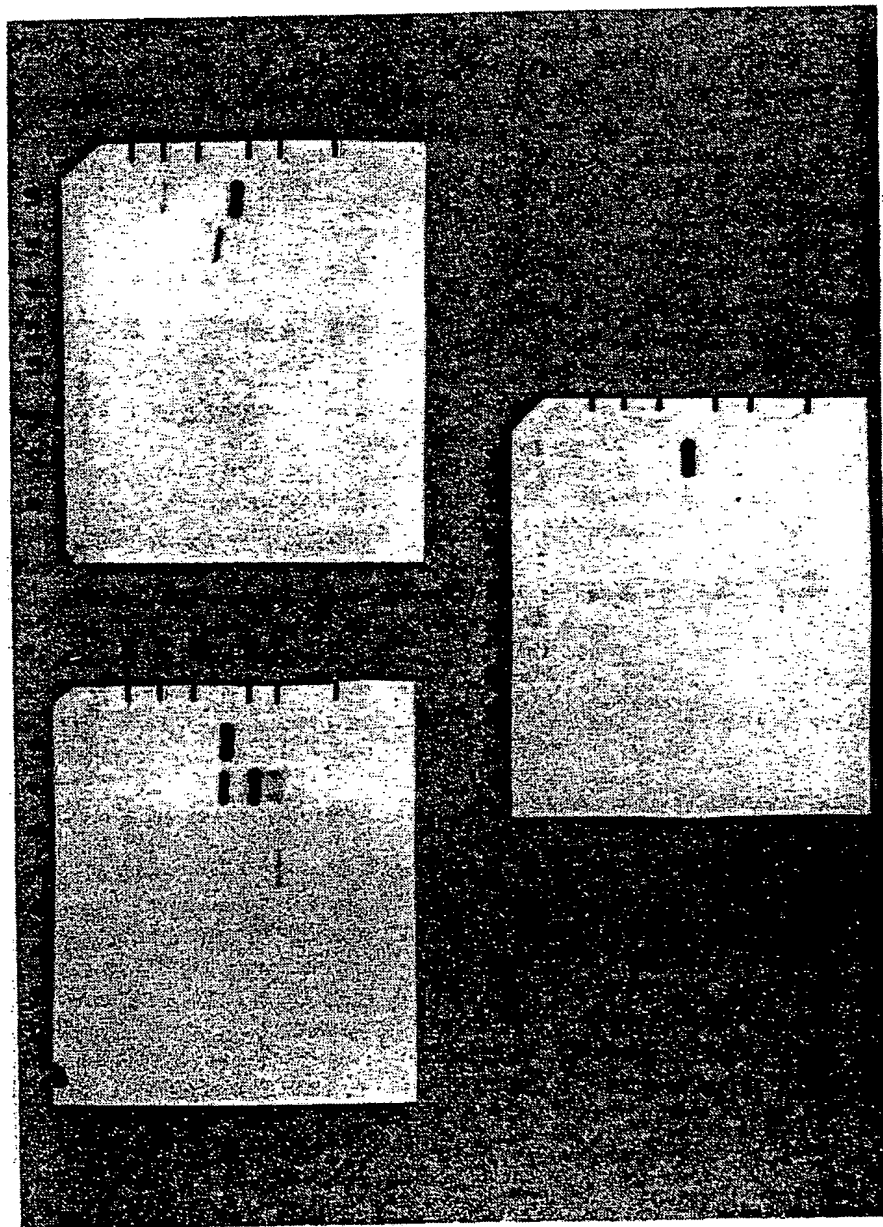


FIG. 4.

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CARBONIC ANHYDRASE NUCLEOTIDE PROBE (56-mer)

parotid

liver

sub mandibular

FIG.5.

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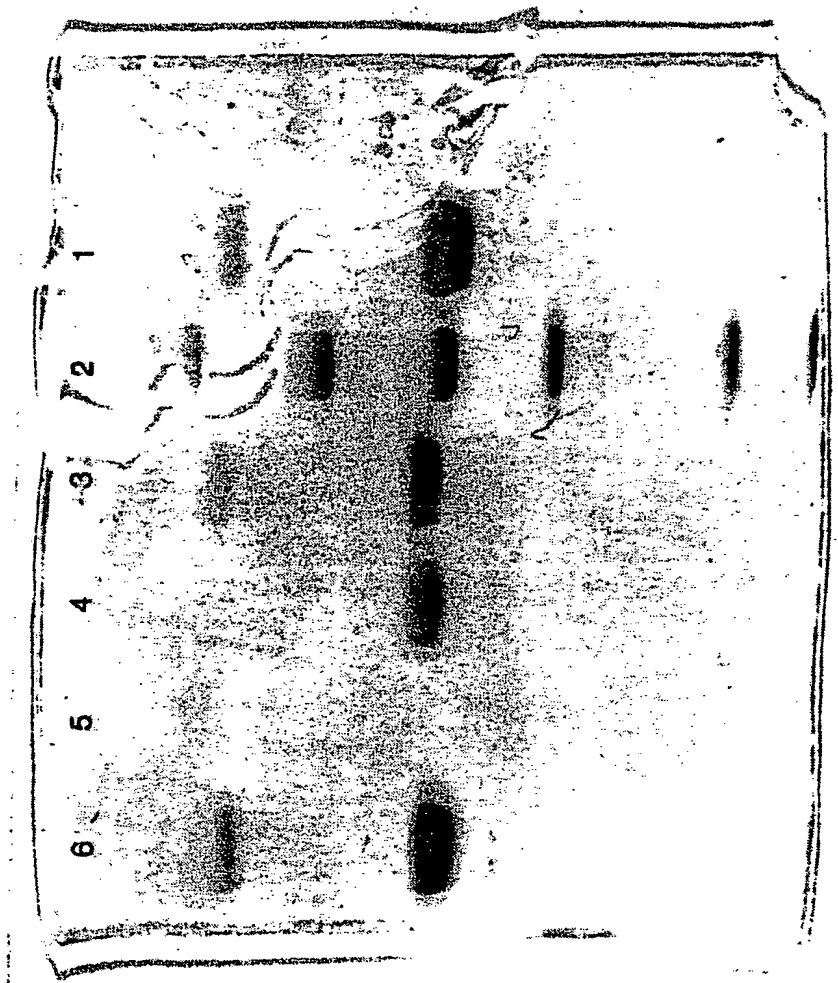


FIG. 6.

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# INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00438

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>1</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <div style="text-align: center; font-size: 1.2em;">Int. Cl.<sup>4</sup>    C12N 9/88</div>						
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; font-size: 0.8em;">Minimum Documentation Searched<sup>2</sup></div> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;">Classification System</td> <td style="width: 50%; border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; text-align: center; padding: 10px 0;">IPC</td> <td style="border: none; text-align: center; padding: 10px 0;">C12N 9/88</td> </tr> </table> <div style="text-align: center; font-size: 0.8em;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched<sup>3</sup></div>			Classification System	Classification Symbols	IPC	C12N 9/88
Classification System	Classification Symbols					
IPC	C12N 9/88					
AU : IPC as above						
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>4</sup></b>						
Category <sup>5</sup> 1	Citation of Document, 1 <sup>1</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. 1 <sup>13</sup>				
X	Annals New York Academy of Sciences Vol 429, 1984 Feldstein, Judith Breiner; Silverman, David N. "Properties of Carbonic Anhydrase from the Saliva of the Rat", pages 214-215	(1)				
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X	Journal of Biological Chemistry Vol 259, No 9, Issued May 10, 1984 Feldstein, Judith Breiner; Silverman, David N. "Purification and Character- ization of Carbonic Anhydrase from the Saliva of the Rat", pages 5447-5453	(1)				
(continued)						
<div style="display: flex; justify-content: space-between; font-size: 0.8em;"> <div style="width: 45%;"> <p>• Special categories of cited documents: 14</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>						
<b>IV. CERTIFICATION</b>						
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.1em;">(05-04-88) 5 APRIL 1988</div>					
International Searching Authority <div style="text-align: center; font-size: 1.1em;">Australian Patent Office</div>	Signature of Authorized Officer <div style="display: flex; align-items: center;"> <div style="text-align: right; font-size: 1.1em;">D.J. SHEPHERD</div> </div>					

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

- X,P Journal of Biological Chemistry Vol 262, No 3 (1)  
 Issued January 25, 1987 Murakami, Hiroshi;  
 Sly, William S. "Purification and Characterization  
 of Human Salivary Carbonic Anhydrase", pages 1382-1388
- X Chemical Abstracts, Volume 101, No 25, issued (1)  
 17 December 1984 (17.12.84) (Columbus, Ohio, USA)  
 Feldstein, Judith Breiner "Purification and  
 characterization of carbonic anhydrase from the saliva  
 and red cells of the rat.", see page 332, the abstract  
 no. 225518d, Diss. Abstr. Int. B 1984, 45(4), 1165

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remarks on Protest:

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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